Regulation of Fas (CD95)-Induced Apoptotic and Necrotic Cell Death by Reactive Oxygen Species in Macrophages

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Although reactive oxygen species (ROS) have long been suspected to play a key role in Fas (CD95)-induced cell death, the identity of specific ROS involved in this process and the relationship between apoptotic and necrotic cell death induced by Fas are largely unknown. Using electron spin resonance (ESR) spectroscopy, we showed that activation of Fas receptor by its ligand (FasL) in macrophages resulted in a rapid and transient production of hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$). The response was visible as early as 5 min and peaked at approximately 45 min post-treatment. Morphological analysis of total death response (apoptosis vs. necrosis) showed dose and time dependency with apoptosis significantly increased at 6 h after the treatment, while necrosis remained at a baseline level. Only at a 35-fold increase in apoptosis did necrosis become significant. Inhibition of apoptosis by a pan-caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (zVAD-fmk), significantly inhibited cell necrosis, indicating the linkage between the two events. Catalase (H_2O_2 scavenger) and deferoxamine ($^{\cdot}OH$ scavenger) effectively inhibited the total death response as well as the ESR signals, while superoxide dismutase (SOD) ($O_2^{\cdot-}$ scavenger) had minimal effects. These results established the role for H_2O_2 and $^{\cdot}OH$ as key participants in Fas-induced cell death and indicated apoptosis as a primary mode of cell death preceding necrosis. Because the Fas death pathway is implicated in various inflammatory and immunologic disorders, utilization of antioxidants and apoptosis inhibitors as potential therapeutic agents may be advantageous.
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The pivotal role of apoptosis in the regulation of physiologic and pathologic conditions is well established (Cohen, 1993; Raff, 1993; Evan and Littlewood, 1998). Apoptosis, which is different from necrotic cell death occurring in response to various stimuli, encompasses a highly conserved series of molecular events termed apoptotic cascade leading to cell shrinkage, chromatin condensation, DNA fragmentation, and ending with cellular disintegration into numerous membrane enclosed apoptotic bodies. The apoptotic bodies become rapidly phagocytosed by neighboring cells and/or professional phagocytes such as macrophages, thereby preventing their eventual disintegration and further tissue injury via leakage of their noxious contents. In view of its physiological role, apoptosis is a two-stage process with an intracellular component representing molecular signal transduction events leading to an intercellular component, characterized by phagocytic clearance of apoptotic bodies. Necrosis is widely believed to be a characteristic of disease conditions and a passive process, characterized by cellular swelling, organelle lysis, and eventual plasma membrane disintegration culminating with the release of cytosolic contents into the environment. While distinct, both forms of cell death have been a focus of intense research with apoptosis leading the way due to its active and regulatable nature.

Dysregulation of either intracellular or intercellular components of the apoptotic process is an established causative and/or contributing factor in CNS degenerative disorders (Mattson, 2000; Vila et al., 2001), cancer (Liao et al., 2001; Reed, 2001; Su et al., 2001; Wolf et al., 2001), and numerous inflammatory conditions ranging from acute systemic insults exemplified by septic shock (Adrie et al., 2001; Feterowski et al., 2001) to localized ones, such as acute respiratory distress syndrome (ARDS) (Fine et al., 2000; Lesur et al., 2000). Given that excessive necrosis has been traditionally viewed as a characteristic of the acute disorders, and the involvement of apoptotic cascades has recently been established, a potential link between the two emerges. Certain apoptotic effectors, such as the Fas/Fas receptor by its ligand (FasL) system, have been proposed to induce both death responses via divergent

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mechanisms. However, the specific mechanisms and their potential linkage remain to be further elucidated.

The Fas/FasL system play important roles in the regulation of the immune system (Siegel et al., 2000; Goodnow, 2001; Hsu et al., 2001) and in various apoptosis conditions such as those evoked by anti-tumor agents, viral infections, and irradiation (Westendorp et al., 1995; Friesen et al., 1996; Muller et al., 1997; Rehemtulla et al., 1997). Induction of apoptosis through Fas involves activation of caspase-8 at the death-inducing signaling complex (DISC). The DISC comprises oligomerized Fas, the adapter FADD/Mort1, and the cysteine protease caspase-8, activation of which initiates the caspase cascade for apoptosis (Boldin et al., 1996; Muzio et al., 1996; Medema et al., 1997). Along with commonly accepted macromolecular effectors of the Fas death pathway, including FADD/caspase-8/Bid/Bax/cytochrome C/Apaf-1/caspase-3 cascade, significant effort has focused towards the role and dominancy of reactive oxygen species (ROS) in the demise of cells following Fas engagement. A byproduct of aerobic metabolism, ROS are produced at the levels of organelles such as mitochondria (Stoian et al., 1996; Cai and Jones, 1998; Raha and Robinson, 2001), and are established participants/regulators of numerous signaling events such as activation of gene transcription, proliferative regulation, antimicrobial defense, and others (Cai and Jones, 1998). Aside from their physiological roles, ROS also participate in the regulation of apoptosis and are often involved in disease conditions via DNA-adduct formation for example (Raha and Robinson, 2001). Also, in response to a range of chemical stimuli ROS outbursts have been suggested to directly lead to necrosis via stimulation of lipid peroxidation and overall disturbance of cellular redox status (Bai and Cederbaum, 2001; Han et al., 2001).

Several studies have attempted to address the specific identity and ultimate role of ROS on the fate of the cell; however, consensus has yet to be reached. Furthermore, the relationship between the apoptotic and necrotic mode of death, which we term "Total Death Response" in the Fas/FasL system remains unaddressed. In a rather imaginative study, Vercammen et al. (1998) suggested a dual pathway resulting in either caspase-mediated cell death with apoptotic morphology or a necrotic one via massive ROS production. Gulbins et al. (1996) indirectly implicated superoxide ($O_2^{\cdot-}$) radical as a functional mediator of the Fas-induced cell death in Jurkat cells, while Hug et al. (1994) found no requirement of reactive oxygen intermediates in L929 murine fibroblasts stably expressing human Fas. Several other studies along with Gulbins et al. (1996) (Um et al., 1996; Deas et al., 1997) analyzed intracellular ROS generation via flow cytometry using dichlorofluorecein diacetate as a fluorescent probe, presuming the specific reactivity of the probe with H_2O_2 (Bass et al., 1983). Subsequent studies showed the probe's reactivity to represent overall oxidative stress and not the H_2O_2 specific one (Cathcart et al., 1983). More recently, O'Malley et al. (2004) demonstrated that oxidation of the fluorescent probe can occur without ROS generation. The general leakiness of the probe was also suggested, thereby potentially risking underestimation or even failure of ROS detection. To circumvent these problems, we utilized a highly specific and sensitive electron spin resonance (ESR) spectroscopy to identify and quantify specific oxygen species generated during Fas ligation. Various inhibitors of ROS were used to determine the role of specific ROS in Fas-mediated cell death. Macrophages were used as a model cell system since they are a major source of ROS production and are key participants of the immune system.

MATERIALS AND METHODS

Cells and reagents

The macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Rockville, MD) and was used throughout. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Life Technologies, Gaithersburg, MD) supplemented with 5% fetal calf serum (FBS), 2 mM glutamine, and 100 U/ml penicillin–streptomycin. Recombinant FasL (*Super*FasL) was obtained from Alexis Biochemicals (San Diego, CA). Benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (zVAD-fmk), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), and deferoxamine were obtained from Sigma (St. Louis, MO). Superoxide dismutase (SOD) and catalase were purchased from Boehringer Mannheim (Indianapolis, IN).

ESR

ESR spin trapping technique was used to detect short-lived free radical intermediates. This technique involves the addition-type reaction of a short-lived radical with a paramagnetic compound as a spin trap (DMPO was used as a spin trapping agent) to form a relatively long-lived free radical product (spin adduct), which can then be studied using conventional ESR. The intensity of the signal is used to measure the amount of short-lived radicals trapped, and the hyperfine couplings of the spin adduct are generally characteristics of the original trapped radicals. The spin trapping is a method of choice for detection and identification of free radical generation because of its sensitivity and specificity. All ESR measurements were conducted using a Varian E9 ESR spectrometer and a flat cell assembly. Reactants were mixed in a test tube in a final volume of 500 μl. The reaction mixture was then transferred to a flat cell for measurement. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) as reference standards. The software EPRDAP, version 2.0, was used for data acquisition and analysis.

DNA fragmentation ELISA assay

Cells were plated in a 96-well plate at a density of 1×10^5 cells/ml 24 h before the cells were subjected to treatment in triplicate wells. After treatment, the cells were washed in phosphate-buffered solution and apoptosis was quantified by measuring the level of histone-bound DNA fragments using an ELISA assay kit (Boehringer Mannheim), according to the manufacturer's instructions. Briefly, the cells were lysed with 200 μl of lysis buffer at room temperature. The cell lysate (20 μl) was mixed with an antibody solution (80 μl), and allowed to incubate at room temperature for 2 h. The substrate was added after the wells were washed three times with a washing buffer. After incubation for 10 min at 37°C, the reaction was stopped, and optical density was measured using a microplate reader at a wavelength of 405 nm.

Morphological analysis of cell death

Morphological analysis of cell apoptosis was performed using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) kit (Boehringer Mannheim). After treatment, cells were washed in phosphate-buffered solution and fixed in 4% paraformaldehyde at room temperature for 5 min. The cells were then permeabilized and incubated in a reaction medium containing terminal deoxynucleotidyl transferase and fluorescein-dUTP for 60 min at 37°C. The cells were counterstained with propidium iodide and examined under a fluorescence microscope. A bright green fluorescence signal representing fragmented DNA indicated TUNEL-positive apoptotic cells. Cell necrosis was determined by trypan blue exclusion assay. Trypan blue stained only the cells with disrupted plasma membrane, thereby indicating a necrotic stage of cell death. In some experiments, propidium iodide assay was also used to confirm necrotic cell death. All experiments were performed at 37°C in 48-well plates at a

density of 1×10^5 cells/ml. A minimum of 10 fields of view was analyzed for each treatment.

Statistical analysis

The numeric data are presented as mean ± SD of 4–6 separate experiments. The difference between data groups and controls was determined using a Student's *t*-test. A *P* value below 0.05 was considered statistically significant.

RESULTS

Induction of apoptosis by FasL

To address the question of the role of ROS in Fas-mediated cell death, we first characterized the dose/time effects of FasL on apoptotic cell death using ELISA-based DNA fragmentation assay. An approximately 3-fold increase in apoptosis level was detected at 0.1 μg/ml with the response exceeding 10-fold increase at 0.5 μg/ml (Fig. 1A). Significant apoptotic response was observed as early as 6 h and peaked at about 15 h post-treatment (Fig. 1B). To provide morphological evidence of apoptosis, cells were similarly treated with FasL and subjected to TUNEL assay at 12 h post-treatment. Indeed, green aggregates, representing fragmented DNA, can be clearly visualized (Fig. 1D), as opposed to control showing no green fluorescent staining (Fig. 1C). Note that the nuclei of apoptotic cells shared a round shape while the healthy cells maintained a more extended one, stemming from their attachment to the plate's surface.

ESR characterization of FasL-induced ROS response

To investigate the role of ROS as mediators of Fas-mediated cell death, ESR studies using the spin trap DMPO were carried out. Cells were treated with FasL (0.5 μg/ml) and immediately transferred to a flat cell assembly for ROS measurements. DMPO was verified to be free of contamination and the DMPO-treated cells served as negative controls (Fig. 2A,B). In response to FasL treatment, a clear positive signal was observed (Fig. 2C). The ESR spectrum consists of a 1:2:2:1 quartet with hyperfine splittings of $a_H = a_N = 14.9$ G, where a_N and a_H denote hyperfine splittings of the nitroxyl nitrogens and α-hydrogen, respectively. Based on these splittings and the 1:2:2:1 line shape, the spectrum was assigned to the DMPO-˙OH adduct, which is evidence of ˙OH generation. To characterize the kinetics of the response, ESR spectra were measured over the period of 60 min and peak heights were recorded (Fig. 2D). Hydroxyl radical generation was detectable as early as 5 min, while the peak response was reached at approximately 45 min post Fas engagement.

Effect of ROS scavengers on FasL-induced ROS generation

To confirm the identity of the observed free radical, ROS inhibition studies were carried out. RAW 264.7 cells were treated with FasL in presence or absence of specific ROS scavengers (Fig. 3). The addition of catalase completely inhibited the signal intensity, indicating that H_2O_2 was generated in FasL-treated cells and that this oxidative species was a precursor for ˙OH generation, i.e., through a Fenton-like reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$). The observed protective effect of catalase was consistent with previous reports identifying it as a major contributor to the regulation of cellular redox status (Huang et al., 2000; Bai and Cederbaum, 2001; Isuzugawa et al., 2001; Wang et al., 2003). Addition of deferoxamine (˙OH scavenger) also decreased the signal intensity, further supporting the ˙OH generation. SOD ($O_2^{\cdot-}$ scavenger) showed weak inhibitory effect on the ESR signal.

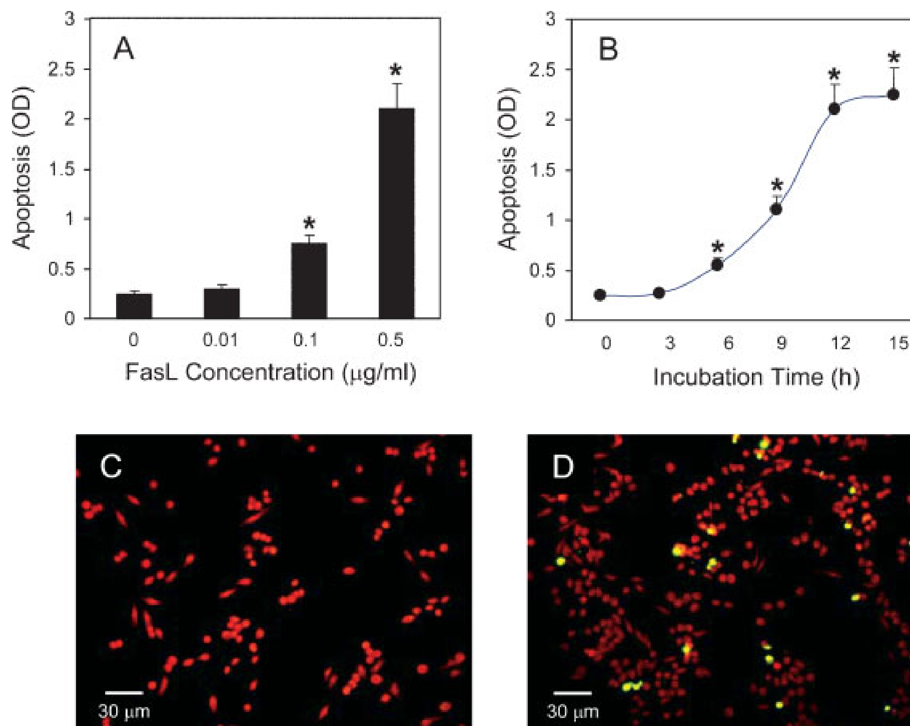

Fig. 1. Dose and time dependence of apoptosis in response to Fas receptor by its ligand (FasL) treatment. **A**: RAW 264.7 cells (1×10^5/ml) were treated with various doses of FasL (0–0.5 μg/ml) at 37°C for 12 h and analyzed for apoptosis by ELISA. **B**: Time course of apoptosis induced by FasL (0.5 μg/ml). Data are shown as the mean ± SD of four separate experiments. *$P < 0.05$ versus untreated control. **C** and **D**: TUNEL analysis of apoptotic cells at 12 h post-treatment with buffer control or FasL (0.2 μg/ml), respectively. Apoptotic cells are indicated by bright yellow/green fluorescence signal. Red fluorescence signal indicates propidium iodide counterstain.

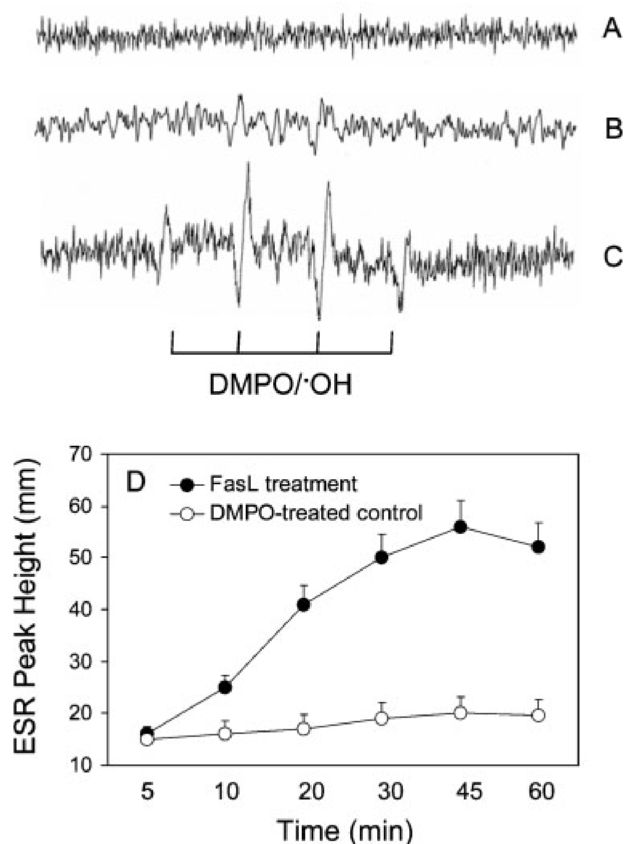

Fig. 2. Electron spin resonance (ESR) characterization of FasL-induced reactive oxygen species (ROS) generation. ESR spectra were recorded at 30 min following the addition of (**A**) the spin trapper 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) (100 mM) alone, (**B**) DMPO (100 mM) + RAW 264.7 cells (1×10^5/ml), (**C**) DMPO (100 mM) + RAW 264.7 cells (1×10^5/ml) + FasL (0.5 μg/ml). The spectrometer settings were as follows: receiver gain at 1.5×10^5, time constants at 0.3 sec, modulation amplitude at 1.0 G, scan time at 4 min, magnetic field at $3{,}470 \pm 100$ G. **D**: Kinetics of the cellular response to FasL was measured by determining the peak intensity from 5 to 60 min post-treatment.

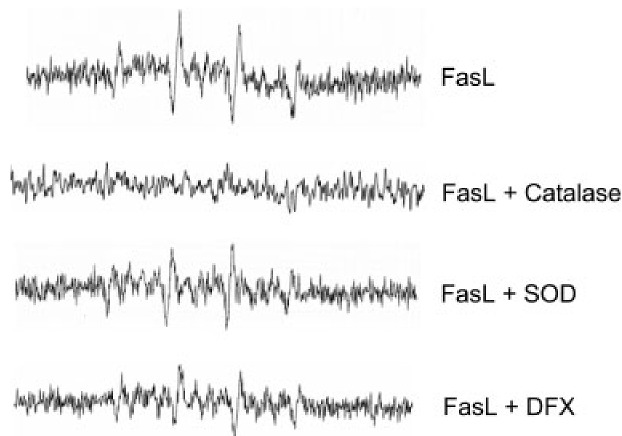

Fig. 3. Effect of ROS scavengers on FasL-induced ROS generation. ESR spectra were recorded 30 min following the addition of FasL (0.5 μg/ml) and DMPO (100 mM) to RAW cells (1×10^5/ml) in the presence of one of the following agents: catalase (1,000 U/ml), superoxide dismutase (SOD) (1,000 U/ml), and deferoxamine (1 mM). Spectrometer settings were the same as in Figure 2.

Differentiation of the mode of cell death following FasL treatment

Our characterization of the total death response to Fas activation was based on morphological assessment using the TUNEL assay as the apoptotic marker and trypan blue exclusion as the necrotic one. Such comparison allowed for accurate determination of the total death response in the system given that TUNEL positive cells represented nuclei with fragmented DNA, a hallmark of apoptosis, and trypan blue positive cells exhibited loss of plasma membrane integrity, a functional characteristic of necrosis. At this point, it should be noted that DNA fragmentation is an active process and as such, not consistent with necrosis where the entire cellular contents are released and dispersed in the environment resulting from the functional collapse of the plasma membrane.

Figure 4A shows percentages of cells exhibiting either apoptotic or necrotic morphology in response to varying doses of FasL. Note that at the concentrations of 0.1 and 0.5 μg/ml significant levels of apoptosis as well as necrosis can be detected. These findings are consistent with those of Jayanthi et al. (1999). However, in both cases a significantly higher component of the cell population exhibited the apoptotic morphology. To determine the potential linkage between apoptotic and necrotic cell death induced by FasL, we assessed the kinetics of the two events. Figure 4B shows that at 6 h post-treatment, no significant necrosis versus baseline level was observed. In contrast, apoptosis increased about 10-fold at this time point. Only at 12 h, with approximately 35-fold increase in apoptosis versus baseline, did the necrosis level become significant. To further confirm the trypan blue results, propidium iodide assay was used to determine necrotic cell death. Figure 4C shows that during the first 6 h of treatment, no significant increase in propidium iodide-positive cells was observed over the baseline level, and only at 12 and 15 h post-treatment did the levels become significant. These results are consistent with the trypan blue results and strongly suggest that necrosis is the secondary effect stemming from the elevated apoptotic levels.

Secondary necrosis of apoptotic cells

To confirm the existence of secondary necrosis, cells were treated with FasL in the presence or absence of caspase inhibitor zVAD-fmk and the levels of apoptosis and necrosis were determined. Figure 5A shows that treatment of the cells with zVAD-fmk effectively inhibited both apoptotic and necrotic cell death caused by FasL. These results along with the kinetics studies indicate that apoptosis was the primary mode of cell death and that necrosis came secondary and as a consequence of apoptosis.

Effect of ROS scavengers on total death response

Having determined the FasL-induced death response and the major ROS produced, we evaluated the effect of a number of specific ROS scavengers on the total death response in RAW 264.7 cells (Fig. 6). Consistent with previous reports in non-Fas systems (Huang et al., 2000; Wang et al., 2003), our results show that catalase, a scavenger of H_2O_2, was able to inhibit both apoptotic and necrotic cell death by FasL. Similar protective effects were also observed with deferoxamine, a $^{\cdot}OH$ scavenger. In contrast to previous reports, suggesting the role of $O_2^{-\cdot}$ in the Fas death signal (Gulbins et al., 1996; Jayanthi et al., 1999), we observed no significant effects by SOD (Fig. 6). Consistent with the initial profile presented in Figure 4, the segment of the population

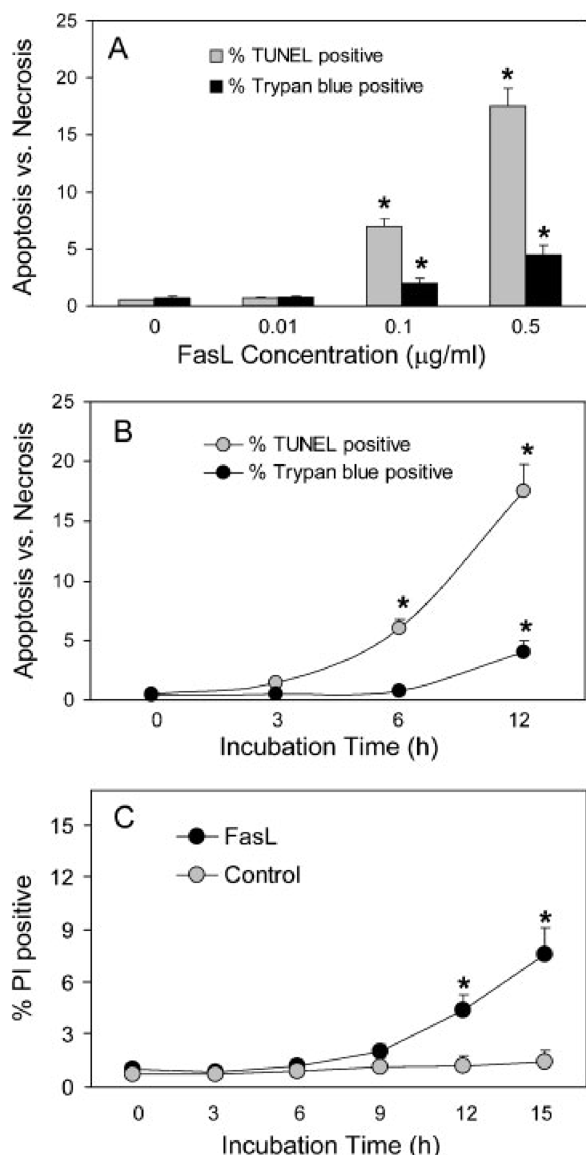

Fig. 4. Differentiation of the mode of cell death following FasL treatment. **A**: Levels of apoptosis and necrosis were determined by TUNEL and trypan blue exclusion at 12 h post-treatment. Note the dose dependent increase in cells exhibiting DNA fragmentation as well as loss of plasma membrane integrity, with the apoptotic ones representing a larger segment of the population. **B**: Time course of the population response. In contrast to apoptosis, no significant difference in necrosis versus control was observed at 6 h. Both responses were significant at 12 h post-treatment (0.5 μg/ml). **C**: Necrosis determined by propidium iodide assay in response to similar FasL treatment. The number of necrotic cells was determined microscopically by nuclear fluorescence staining. Data are shown as the mean ± SD of four separate experiments. *$P < 0.05$ versus control groups.

exhibiting apoptotic morphology significantly exceeded that of a necrotic one in all treatments. This trend maintained during the inhibitory effects of catalase and deferoxamine, thereby strongly arguing that apoptosis and necrosis in the Fas/FasL system are, in fact, linked, and not independent of each other as previously suggested.

DISCUSSION

The findings of this study support the role of ROS, specifically H_2O_2 and $^{\cdot}OH$, in Fas-mediated cell death. Catalase, a constituent of cellular redox defenses

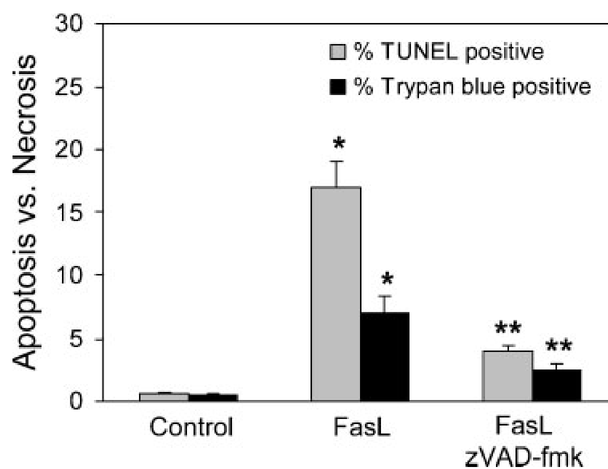

Fig. 5. Cell death response to caspase inhibitor. Cells (1×10^5/ml) were pretreated with benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (zVAD-fmk) (50 μM) for 1 h and the total cell death response to FasL (0.5 μg/ml) was determined after 12 h. Values are mean ± SD; n = 4. *$P < 0.05$ versus untreated controls. **$P < 0.05$ versus FasL-treated control groups.

showed clear inhibitory effects against ROS production and total death response. These findings are consistent with numerous reports identifying catalase's anti-apoptotic effects in response to various stimuli ranging from xenobiotics to cytokines (Huang et al., 2000; Bai and Cederbaum, 2001; Isuzugawa et al., 2001; Wang et al., 2003). In contrast to previous reports (Gulbins et al., 1996; Jayanthi et al., 1999), however, SOD showed minimal protective effects in our system. This was likely a reflection of different models and methodologies used. ESR is a preferred method for identifying specific ROS offering a high degree of specificity in contrast to chemiluminescence and dye based methods used in previous studies. Our ESR studies showed a fractional decrease of the $^{\cdot}OH$ signal following SOD treatment which was somewhat unexpected given that SOD

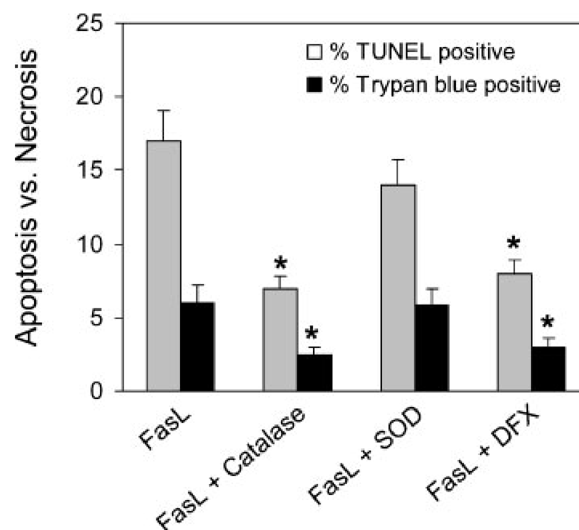

Fig. 6. Effect of ROS scavengers on FasL-induced apoptotic and necrotic cell death. Cells (1×10^5/ml) were pretreated with catalase (1,000 U/ml), SOD (1,000 U/ml), or deferoxamine (1 mM) for 1 h and the total death response to FasL (0.5 μg/ml) treatment was determined after 12 h. Values are mean ± SD; n = 4. *$P < 0.05$ versus FasL-treated control groups.

converts $O_2^{\cdot -}$ to H_2O_2, which is further converted either to H_2O via catalase or glutathione peroxidase, or to $^{\cdot}OH$ via metal-dependent Fenton-like reactions. In good agreement with previous reports (Huang et al., 2000; Wang et al., 2003), the metal chelator deferoxamine showed protective effects in our model, indicating an important role of the $^{\cdot}OH$ radical. It is possible that phenotypic characteristics of RAW 264.7 determine the degree of sensitivity to specific stimuli (Isuzugawa et al., 2001). Should the level of SOD be high, most of the $O_2^{\cdot -}$ radicals would be efficiently converted to H_2O_2 thereby putting pressure on the catalase and glutathione peroxidase system. It is also conceivable that potential sub-optimal expression levels of catalase could have resulted in prolonged H_2O_2 presence and shift of the redox equilibrium towards excessive $^{\cdot}OH$ production. Such elevated $^{\cdot}OH$ levels, detected in our ESR experiments, could in turn initiate a multitude of divergent pathways leading to execution stage of apoptosis including lipid peroxidation, cytochrome c release, and generation of apoptotic second messengers such as ceramide.

Extreme levels of $^{\cdot}OH$ generated as a result of high dose and likely, non-physiological level stimuli could initiate loss of plasma membrane integrity via excessive lipid peroxidation thereby resulting in the necrotic mode of death. Whether such mechanism plays a role in vivo during the progression of previously discussed acute conditions is unclear. Based on the kinetic assessment of the total death response in our model, specifically the observation that necrosis levels did not become significant until 12 h after the treatment while apoptosis increased rapidly as early as 6 h, it is tempting to speculate how the effects of such kinetic relationship, apoptosis to necrosis, might result in toxicity (a term traditionally synonymous with necrosis) in cells and tissues challenged by excessive apoptosis of their cellular constituents and compromised ability of the macrophages to promptly clear them (Fadok, 1999).

In addition to the kinetic relationship between apoptosis and necrosis, the results of this study further support the linkage between the two events in Fas-mediated cell death. Necrosis developed as a consequence of apoptosis rather than a direct necrotic effect by FasL. This conclusion is supported by the observation that inhibition of apoptosis by zVAD-fmk also inhibited cell necrosis. Furthermore, scavengers of ROS that inhibit apoptosis (catalase and deferoxamine) similarly inhibited necrosis whereas those that do not (SOD) had no protective effect. The results of this study also support the concept that necrosis develops as a result of inefficient or impaired clearance of apoptotic cells, i.e., due to the overwhelming presence of apoptotic signals. Clearance of apoptotic cells by phagocytosis has been shown to be a key event in the apoptosis process (Fadok, 1999; Savill and Fadok, 2000). In vivo, apoptotic cells are engulfed by neighboring cells or phagocytic macrophages (Savill and Fadok, 2000; Wang et al., 2003). As they become apoptotic, cells undergo changes in the composition of their surface, which allows their recognition by phagocytes and subsequent removal. Because removal occurs before cell lysis, the release of intracellular toxic contents into the surrounding tissue is avoided. As a result, apoptotic cell clearance is believed to represent a critical process in the control and resolution of tissue inflammation and injury.

Tremendous advances in the field of apoptosis over the past years have established therapeutic utility in modulation of apoptosis. In the case of sepsis, ARDS, myocardial infarction, stroke, and even idiopathic pulmonary fibrosis—all conditions implicating the Fas/FasL system, caspase inhibitors are leading the way as rational drug design agents. The findings of this study also suggest the potential utility of antioxidants for the management of pathologies characterized by excessive apoptosis, and highlight, at least in terms of traditional understanding, the paradoxical relationship between apoptosis and necrosis. Further understanding of cytotoxic mechanisms regulating cell fate during the progression of disease is crucial as we progress towards the development of more effective therapeutic strategies.

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